

Review of *Nucleophaga* (a primitive, ‘cryptomycotan’ genus): Summary of named and unnamed species, with discussion of contemporary and historical observations

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ABSTRACT

Genus *Nucleophaga* is a parasite of the nucleus of various protozoa, i.e., a number of amoebae (free-living and parasitic forms), types of flagellates, and allegedly certain ciliates. *Nucleophaga* was, since its original description (Dangeard, 1895), considered to be fungal (a Chytridiomycete). Within Chytridiomycetes, the genus was included in family Olpidiaceae (Sparrow, 1960; Karling, 1977) along with a similar genus, *Sphaerita* (occurring in the cytoplasm rather than the nucleus), and with *Olpidium* (some species of which have been more recently determined, cf. James et al. 2006, to have membership in groups other than Chytridiomycota). Recent work (e.g., Corsaro et al., 2014, 2016) has indicated, however, that closer relationships of *Nucleophaga* are with Cryptomycota (‘Rozellomycota’ of some), and perhaps even more so with Microsporidia (Bass et al., 2018). In any case, only two species of *Nucleophaga* (*N. amoebae* and *N. terricolae*) have been examined in molecular and electron-microscopic studies. And, no single publication has yet fully accounted for all named species of the genus and a number of putative species remaining unnamed. Part of the reason for the relatively poorly known status of potential taxa of *Nucleophaga*, among mycologists, is that much early publication on the genus was in protozoological (rather than mycological, or botanical) literature--related to the fact that early observers of *Nucleophaga* were often interested in protozoa as primary subjects of investigation. The purpose of our study is to review literature (including that of protozoology) and bring named species of *Nucleophaga* together, and, importantly, provide discussion of each. A further goal is to assemble an annotated list of potential (unnamed) additional species. We exclude forms once considered to possibly belong to *Nucleophaga*, but which, in all probability, do not. We hope our collation of information will encourage additional finds (and molecular and electron microscopic investigations) of the genus--now understood, along with related genera, to be significant in understanding early fungal/protist (holomycotan) evolution. Published on-line www.phytologia.org *Phytologia* 101(1): 1-18 (March 21, 2019). ISSN 030319430.

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As part of a growing interest in what has come to be known as the ‘holomycotan assemblage’ of organisms (Fungi and protistan relatives), increasing attention has been directed to groups--such as the Cryptomycota (‘Rozellomycota’ of some authors), the Chytridiomycota, the Microsporidia, the Aphelidea and the Choanozoa--which may provide further insights into connections between, and evolution of, primitive fungi (e.g., the Rozellida) and primitive animals (e.g., types of ‘protozoa’ such as Nucleariids). Increasing focus (especially molecular study) is being given to those organisms, among primitive ‘holomycotans,’ that are poorly understood or the systematic placement of which has remained uncertain--in an attempt to fill in gaps in present knowledge and provide missing pieces of the puzzle of early eukaryotic diversification (e.g., Corsaro et al., 2018). There are many such poorly known, primitive organisms (of various types), a number being parasitic; most are in need of taxonomic summaries, where available information (even if scattered or incomplete) is compiled in a given study. Our present summary focuses on one genus, *Nucleophaga*--described by Dangeard (1895)--in part because of the recent attention two of its species have received (Corsaro et al., 2014, 2016), but also because its existence (even if not known by name, for a time) has been ‘documented’ over many years (e.g., see Kirby’s, 1941, mention of work by Carter, 1863 and Greeff, 1866). Following Dangeard’s (1895) formal description of

Nucleophaga, there has been a long subsequent history of observation of the genus (particularly in literature of protozoology), as will be evident in our presentation.

Obviously, the recovery of living examples of poorly known forms of any of the above groups (Chytridiomycota, Cryptomycota, Microsporidia, etc.) or the discovery of new forms--for molecular study--is of high value in evolutionary investigations. A recent example of this type of study (molecular analysis of obscure holomycotans) is that of Corsaro et al. (2014) who indicated finding, and provided sequence information for, *Nucleophaga amoebae* (a nuclear parasite of a type of amoeba)--originally described, as noted, by Dangeard (1895). This study of Corsaro et al. (2014) was founded in part on the morphological (electron-microscopic) work of Michel et al. (2009a). These investigations underscored the importance of such updated studies. *Nucleophaga*, traditionally considered a member of the Chytridiomycota (family Olpidiaceae), was determined to constitute a distinct lineage, related to types of Microsporidia and also (if somewhat more distantly) to genus *Rozella*. *Nucleophaga amoebae* appeared to represent a 'missing link,' or evolutionary 'intermediate step,' within the Cryptomycota.

The stated "rediscovery" (see Corsaro et al., 2014, title page) of the parasite, *Nucleophaga amoebae*, was in the free-living amoeba, *Thecamoeba quadrilineata*--found in a "moss meshwork" (Michel et al., 2009a). Corsaro et al. (2016) soon described a second species of *Nucleophaga*, parasitizing another free-living amoeba (*Thecamoeba terricola*), found on sycamore bark. This second *Nucleophaga* (Corsaro et al., 2016) was determined to be morphologically and genetically similar to, but nonetheless distinct (especially based on molecular analysis) from, *N. amoebae* (cf. Michel et al., 2012; Corsaro et al., 2016). Corsaro et al. (2016) named this second *Nucleophaga*, *N. terricolae*. These two *Nucleophaga* species (*N. amoebae* and *N. terricolae*) were considered (Corsaro et al., 2016) together to constitute a distinct, 'sister' lineage within a general group of microsporidian organisms.

The two *Nucleophaga* species above, *N. amoebae* ('recovered,' Corsaro et al., 2014) and *N. terricolae* (newly described; Corsaro et al., 2016)--along with one additional species, *N. ranarum*, described years before (Lavie, 1935b) from amoebae found in Amphibians--were, until recently, the only three *Nucleophaga* species (species names) listed in Index Fungorum (IF, an updated listing of fungal names). Presently, five names are listed in IF (including the addition of *N. intestinalis* (Brug, 1926) and *N. peranemae* (Hollande & Balsac, 1941); however, a sixth species, *N. hypertrophica* (Epstein, 1922), is not included (as of this writing). In any case, additional potential species of *Nucleophaga* have been described (though not named), a subject of our investigation. Additional forms of *Nucleophaga*--or related organisms--are important to phylogenetic investigation, since such may place near the base of fungal radiation (Bass et al., 2018), and relate to evolution of primitive protozoa (Corsaro et al., 2018).

A number of reviews (several early reviews are summarized by Kirby, 1941) of potential hosts of *Nucleophaga*, and related organisms, are helpful in understanding possible taxa of *Nucleophaga*. This is especially true since *Nucleophaga*--though from the start (Dangeard, 1895) considered to be essentially 'fungal' (for many years viewed as 'chytridiaceous') in character--occurs in (parasitizes) a number of different amoebae (free-living and parasitic forms), at least two major groups of flagellates (Kirby, 1941), and "ciliates of various kinds" (Calkins, 1933, p. 386)--a statement echoed by Sleight (1989, p. 277). Sleight mentioned "rumen ciliates from mammals" as possible hosts [seemingly for *Nucleophaga*], a suggestion, however, in need of verification. In fact, as regards the Ciliata as a whole, further clarification is required as to precisely *which* genera/species are parasitized by *Nucleophaga*; i.e., specific examples seem elusive. Calkins (1909, his fig. 118A) did, though, produce a convincing illustration of a probable *Nucleophaga* in the macronucleus of *Paramecium aurelia*. Nonetheless, in various 'reviews' of occurrence in the literature (e.g., Sassuchin, 1934; Kirby, 1941; Sleight, 1989), it can often be difficult to be sure whether nuclear (*Nucleophaga*) or cytoplasmic (*Sphaerita*) parasites are being discussed--see, for example, Sassuchin's (1934, p. 216) discussion of "parasitism due to fungi."

In any case, a substantial portion of the information about *Nucleophaga* is to be found in (often older) protozoological, rather than mycological (or botanical), literature (see comments in this regard by Sparrow, 1960, p. 125; and Dick, 2001, p. 407). Helpful reviews (or studies with a related review constituent) include those of Pénard (1905), Mercier (1907), Mattes (1924), Brug (1926), Sassuchin (1934), Brumpt and Lavier (1935), Lavier (1935b), Hollande and Balsac (1941) and Kirby (1941). The strong protozoological component to the literature of *Nucleophaga* accounts in part for why some named species, and additional potential species, of the genus were not always noted in mycological listings, e.g., *Index Fungorum* (IF). A more recent (mycological) review--of *Sphaerita*, *Pseudosphaerita*, *Morella* and *Nucleophaga*--was published by Karling (1972); however, the focus of his paper was to the concepts and potential distinctions of these putative (possibly related) genera, rather than, necessarily, determination of taxa within these genera. Karling's (1977) later coverage of *Nucleophaga* was more sub-generically useful, discussing and illustrating several species. No names for *Nucleophaga* species were added in Longcore's (1996) enumeration. Dick (2001) recognized three species of *Nucleophaga* (accounting for a fourth name, as an alleged synonym)--a listing differing from that of *Index Fungorum*, in that Dick recognized *N. hypertrophica* Epstein (1922).

Hopefully, our listings (below) will provide additional historical and current context of *Nucleophaga*. With the possible exception of *N. amoebae* and *N. terricolae* (listed first, List One)--these being carefully molecularly investigated by Corsaro et al. (2014, 2016, respectively), and well-studied morphologically by Michel et al. (2009a, 2012, respectively)--any of the remaining 'species' (Lists One and Two) should be reinvestigated, since these taxa have not been subjects of molecular or electron microscopic analysis. Such putative taxa, and other primitive fungal parasites of protozoa discovered, would be worth the effort of collecting and studying for possible new insights and connections they may reveal concerning early fungal/protist evolution. We exclude only those forms (List Three)--though at one point suggested to represent *Nucleophaga*--that present scrutiny reveals do not belong in the genus.

TAXONOMIC LISTINGS

We present below three listings: List One is six named, potential species of *Nucleophaga*. List Two is seven, unnamed, but likewise potential taxa of the genus. Presentations are given with attempted fidelity to authors' statements. These lists constitute a more comprehensive, annotated survey of possible species than found elsewhere. List Three is excluded forms. All figures mentioned in text refer to figs. *in* publications of *other* authors, with the exception that our **Figs. 1-19** (these illustrating species from **List One** only) are here redrawn (from the original sources indicated in our three figure-legends).

LIST ONE: The six, named species of *Nucleophaga*. Because it is not certain any of these taxa are identical, we treat them as potentially distinct. Species 1, 2 and 6 parasitize free-living protozoa; whereas, 3, 4 and 5 are 'hyperparasites,' parasitizing protozoa which are potentially parasitic. See our Figs. 1-19 (species #2, morphologically similar to #1 under the light microscope, is not separately illustrated).

1. *Nucleophaga amoebae* Dangeard (1895). The genus *Nucleophaga* was established by Dangeard (1895), as well as the original species, *N. amoebae*. It was discovered by Dangeard in the free-living *Amoeba*, *A. verrucosa*, which Pénard (1905) considered a form of *A. proteus* (see also Kirby, 1941). Useful illustrations of *Nucleophaga amoebae* are Dangeard's (1895) figs. 3 and 4--his fig. 4A (p. 210), for example, seems to be an illustration cited in broad treatments of Chytridiomycetes such as Sparrow (1960, p. 124, fig. 12A) and Karling (1977, p. 31, pl. 12, fig. 9). A distinct sporangial membrane is interpreted to be present in this *Nucleophaga* (see Dangeard's fig. 4D; and see our Discussion, 2nd paragraph); Dangeard's figs. 4B,C,E,F nicely illustrate that more than one [*N. amoebae*] parasite can be present in a given host-nucleus. As for taxonomic evaluation of the host, there is an indication in Hall (1953) that *Amoeba verrucosa* should in fact be *Thecamoeba verrucosa* (see also below).

Corsaro et al. (2014) indicated recouping *Nucleophaga amoebae* (Dangeard, 1895) in the free-living amoeba, *Thecamoeba quadrilineata*--see also Michel et al. (2009a). Micrographs of the *Nucleophaga* in Michel et al. and Corsaro et al. do bear similarity to drawings provided by Dangeard (mentioned above). However, it should also be mentioned here that the amoeba species examined by Michel et al. and Corsaro et al., *Thecamoeba quadrilineata*, is apparently *not* the same species of amoeba examined by Dangeard (viz. *Amoeba verrucosa* = *Thecamoeba verrucosa*; cf. Bovee, 1985); in fact, *Thecamoeba quadrilineata* may be more similar to *T. sphaeronucleolus* than it is to *T. verrucosa* (see the taxonomic account of Bovee, pp. 172-173). Regardless of the question of the absolute certainty of its identity to the organism originally observed by Dangeard (1895), the *Nucleophaga* studied by Corsaro et al. (2014), in *Thecamoeba quadrilineata*, is indeed the first *Nucleophaga* species to be genetically sequenced--a study of unquestioned value in determining the true relationships of genus *Nucleophaga*.

2. *Nucleophaga terricolae* Corsaro et al. (2016). Described by Corsaro et al. (2016), and related to *N. amoebae* (see above), this is the only *other* species of *Nucleophaga* that has been sequenced (and is, thus, presented here next). It was isolated from *Thecamoeba terricola*, a free-living amoeba found on sycamore bark (Michel et al., 2012). Under the light microscope, *N. terricolae* is very similar to *N. amoebae* (and is not here separately illustrated). Corsaro et al. (2016) determined *Nucleophaga terricolae* to be 94.5 % genetically identical (18S rDNA) with *N. amoebae*, forming (together) a distinct clade within various microsporidian organisms--all within a broadly defined (Corsaro et al., 2016) Rozellomycota (= Cryptomycota) including *Rozella*. Though having an appearance and developmental cycle similar to *N. amoebae*, *N. terricolae* showed (under TEM) minor morphological differences--including slightly more polymorphic and amoebic trophont-stages and, additionally, slightly larger, later ('spore') stages--see Corsaro et al. (2016, p. 3005). As for hosts, the amoeba, *Thecamoeba terricola*, in which *N. terricolae* occurs, is a distinct species from the *Thecamoeba* in which Corsaro et al. (2014) studied *N. amoebae* (see Bovee, 1985, who, by the way, noted that "*Nucleophaga*" parasitizes *Thecamoeba terricola*).

At an early date, Mattes (1924) described an organism which he named "*Sphaerita nucleophaga*," later discussed by Karling (1972). Mattes (1924) indicated this organism occurred within the nucleus of the following three *Amoeba* species: "*A. sphaeronucleolus*," "*A. vespertilio*," and (less often) "*A. terricola*." This "*Sphaerita*" of Mattes is, in all probability, *Nucleophaga*--not *Sphaerita*, which would occur in the cytoplasm rather than the nucleus. It is questionable, though, that the apparent *Nucleophaga* organisms in the three different amoeba species are the same; for example, the *Nucleophaga* (cf. Mattes, plate 19, figs. 17-24), as we refer to it here, in *Amoeba sphaeronucleolus* pushed the remains of the nucleolus to the periphery of the nucleus, flattening it as a residual cap (of relatively limited extend); whereas, the putative *Nucleophaga* in *Amoeba terricola* (his plate 20, figs. 32,33,34), apparently, 'marginalized' the chromatin material as a thin layer, spread more or less uniformly around the inner face of the nuclear membrane. Be this a consistent difference or not, one may wonder if Mattes (1924; see his plate 20, e.g., figs. 32, 34) might have been observing, in *Amoeba* (*Thecamoeba*) *terricola*, a species of *Nucleophaga* similar to that described by Corsaro et al. (2016), viz. *N. terricolae*? The tightly packed spore-mass of the nuclear parasite shown in the morphological work of Michel et al. (2012; see fig. 1b, p. 38) on *Thecamoeba terricola*--a basis for the study of Corsaro et al., 2016, in which *Nucleophaga terricolae* was described--is indeed similar to that in fig. 34 (plate 20) of Mattes (1924); the main difference *apparently* is a narrow, 'empty' zone around the spore mass, evident, if not labeled, in fig. 1b, Michel et al. (2012); this zone possibly represents peripheral 'space' where depleted chromatin material, or scattered remnants of the nucleolus (Michel et al., fig. 5b), existed--as such became marginalized, prior to destruction by nucleophagy. In addition to Mattes (1924), even earlier, Pénard (1905, p. 196) may have observed what eventually came to be called *Nucleophaga terricolae* in *Amoeba* (*Thecamoeba*) *terricola*, although Pénard seemingly assumed (due to a generally similar appearance) it was the same as the parasite initially described by Dangeard (1895) in '*Amoeba verrucosa*,' viz. *Nucleophaga amoebae*.

3. *Nucleophaga hypertrophica* Epstein (1922). Epstein, writing in Russian (providing a German summary), described the species, *N. hypertrophica*, found in the human parasitic amoeba, *Endolimax nana* (occurring in the colon and caecum; cf. Manwell, 1961). *Endolimax nana* also occurs in other mammals such as swine and monkeys; *E. nana* is similar in size to, but morphologically distinguishable (particularly in the cyst stage) from, the amoeba discussed under #4 below. As a parasite of a parasite, *Nucleophaga hypertrophica* would, by definition (see Manwell), be a 'hyperparasite.' The name *hypertrophica*, however, indicates enlargement (hypertrophy) of the amoeba nucleus as a result of parasitism by *Nucleophaga*. Figures 1,3,6,7,9,14 in Epstein (1922) clearly indicate this parasite is *Nucleophaga*; a spore of this organism may infect the nucleolus (Epstein, 1922, his fig. 3), subsequently dividing internally (fig. 6 of Epstein, 1922) in a central position and displacing chromatin material outward (this pattern may constitute a difference from *N. intestinalis*, discussed below). Nöller (1921) may have observed what was *N. hypertrophica* a year earlier (see Wenyon, 1926, p. 253; Kirby, 1941, p. 1046), though seemingly not naming it. Epstein (1922) did not appear to base his study and descriptive information on Nöller (1921), Nöller's work not being cited by Epstein. In any case, see discussions concerning Nöller and Epstein in Lavier (1935b) and also Kirby (1941). In Epstein's (1922) fig. 9, there is a hint of a 'clear zone' surrounding the *Nucleophaga* spores--discussed under #4, below.

4. *Nucleophaga intestinalis* Brug (1926). This organism was described as a parasite of a human intestinal amoeba that would eventually be named *Iodamoeba bütschlii* (which has been spelled *I. bütschlii*, cf. Manwell, 1961); it was formerly named *Endolimax williamsi*, cf. Levine (1973). This amoeba is the most common amoeba in swine (Levine, 1973). Brug (1926) studied *Nucleophaga intestinalis* from a prepared slide, a number of examples being observed. This nuclear parasite (aggregation of coccoid spores) was observed to be surrounded by a 'clear zone' or 'space,' rather than a sporangial membrane; Brug (p. 468) thus spoke of the "nakedness" of this *Nucleophaga*, and considered it distinct. In [apparently] lacking a sporangial membrane, there is resemblance to #3, above, *Nucleophaga hypertrophica* (Epstein, 1922; see Kirby, 1941, p. 1046). Brug (1926), though, seemed unaware of Epstein's work. Brumpt and Lavier (1935) considered Brug's species (*intestinalis*) a synonym of Epstein's species (*hypertrophica*), as did Dick (2001). Nonetheless, Karling (1977) appeared to recognize Brug's species. An illustration of what is probably *N. intestinalis* is in Kudo (1966, p. 1074, fig. 387b). It is our belief that, since the host amoebae originally studied by Epstein (1922) and Brug (1926) were distinct genera, there is insufficient basis to be certain the respective *Nucleophaga* species infecting them were the same--regardless of subsequent statements (Brumpt and Lavier, 1935; Epstein, 1935; Kirby, 1941)--especially in the absence of molecular data. These two hyperparasites could prove to be different species, regardless of similar appearance; *N. intestinalis* appears to push and flatten the nucleolus outward, rather than directly infecting it within (as in *N. hypertrophica*); in either case, the nucleolus ultimately disappears (due to probable nucleophagy).

5. *Nucleophaga ranarum* Lavier (1935b). This species of *Nucleophaga* was found parasitizing *Entamoeba ranarum* occurring in intestines of tadpoles of the European Midwife Toad, *Alytes obstetricans* (see Lavier, 1935b). As a parasite of a parasite, this *Nucleophaga* would be considered (as in #3 and 4 above) a 'hyperparasite.' *Nucleophaga ranarum* is noted by Index Fungorum (IF), but not by Dick (2001). Because the *N. ranarum* life cycle seemed a combination of the two types of development said to occur in *Nucleophaga* -- see though Kirby, 1941, who thought the *Nucleophaga* described by Lavier (1935b) could represent a 'type one' development; but, contrastingly, see our Discussion, second paragraph -- Lavier (1935b) considered this species distinct, providing a species name, *N. ranarum*. This species seems unusual in that, at a very young stage, a plasmodial-like structure appears to engulf the nucleolus and adjacent portions of the nucleus (Lavier's, 1935b, fig. 6, pl. 10). Though first mentioned (but not named to species) in Lavier (1935a), Lavier (1935b) published the more complete account of this species later that year--both publications in French, and in parasitological/protozoological literature. If the publication (1935b) is deemed 'protozoological,' one might assume the organism described would have been named in accordance with the zoological code of nomenclature. However, since *Nucleophaga* was, from its inception (Dangeard, 1895), considered a Chytridiomycete (therefore fungal in nature)--and since

Lavier (1935b) also considered it a chytrid--it should have been named in accordance with the botanical code pertaining at the time (fungi being regarded, *for nomenclatural purposes*, as 'plants'). This might seem a relatively moot point, except that the botanical code, by then, *required* a diagnosis *in Latin* (beginning Jan. 1, 1935, i.e., the year in which *N. ranarum* was subsequently described). Since no Latin diagnosis was given by Lavier (1935a or 1935b), a diagnosis (in Latin *or* English; cf. McNeill et al., 2012) would need to be provided if it were desired to formally validate this name.

6. *Nucleophaga peranemae* Hollande & Balsac (1941). This species was discovered as a nuclear parasite in *Peranema trichophorum* (a free-living, heterotrophic, colorless euglenoid organism). Since this species was found in a protozoan (euglenoid flagellate) other than an amoeba, and since it was observed to form a (minutely spiny) resting spore or cyst (see pp. 38, 41, and fig. 14 in Hollande & Balsac)--a resting spore not otherwise reported in *Nucleophaga*--this parasite was considered to be a new species of *Nucleophaga*. Hollande and Balsac placed value (with which we agree), in determining species, on the host occupied, this host being quite different from those previously reported for *Nucleophaga*. The development of this species appears to correspond to 'type-one' listed in Kirby (1941)--see 2nd paragraph of our Discussion--although this species was not covered by Kirby (1941) since Kirby's was a contemporaneous publication. Hollande and Balsac's (1941) illustrations (e.g., their fig. 6) of the sporangium indicate that the parasite develops as a plasmodium with a surrounding membrane (although this is not necessarily clear from their text discussion). It is perhaps worth noting that, contrary to Hollande and Balsac's claim, theirs is not the first mention of occurrence of *Nucleophaga* in a flagellate (i.e., in protozoa other than amoebae). Kirby (1940), in another publication, noted finding *Nucleophaga* in a different type of flagellate--*Caduceia theobromae*--a 'Devescovichid' flagellate (see #6, List Two), such flagellates occurring in the digestive tract of termites. *Nucleophaga peranemae* was recognized by Dick (2001, p. 407); as noted by Dick, though, validation of this name would require supplying a proper diagnosis.

LIST TWO: Probable additional species of *Nucleophaga* (not named), given here as "*Nucleophaga* sp." Since there have been many observations of *Nucleophaga* over time, our listing will surely prove incomplete; further possible species are mentioned in the last paragraph of our Discussion.

1. *Nucleophaga* sp. (see Tyzzer, 1920). Tyzzer (1920) described, as new, a relatively small species of amoeba--*Pygolimax gregariniformis*--occurring in the caecum of young turkeys (and apparently also chickens). In nuclei of some of these amoebae, he found (by means of stained slides) small, more or less coccoid inclusions that had pushed the 'karyosome' (nucleolus) to one side; he also found that the nucleus often became somewhat hypertrophied. In some instances, the nuclear membrane ruptured--the remains of the nucleolus frequently still visible for a time after this occurred. Tyzzer mentioned *Nucleophaga* as a possibility for the organism (nuclear parasite) he found, and noted similarity to Dangeard's species, *N. amoebae*. However, Tyzzer also noted that the coccoid bodies he observed in the protozoan material from turkeys were somewhat smaller (0.4 to 0.8 μm) and not uniformly spheroidal, as compared with those in Dangeard's material (where the coccoid bodies were from 1 to as much as 2.5 μm , and consistently spheroidal). Tyzzer did not apply a species name to his organism, though obviously wondering if it were different (based on size and morphology, and occurring as it did in a distinct host). Certain statements by Tyzzer, and his figures 12-14 (plate 22), appear to confirm this organism as a *Nucleophaga*, possibly a different species--in any case, an organism well worth reinvestigating.

2. *Nucleophaga* sp. (see Kirby, 1927). In describing a new species of amoeba (*Endamoeba disparata*)--occurring in the intestine of a termite (*Mirotermes hispaniolae*) found on Barro Colorado Island, the Canal Zone, Panama--Kirby (1927) noted and illustrated (his figs. 40-45, plate 24) a *Nucleophaga* (not named to species) infecting the nucleus of the amoeba; numerous instances of this parasitism were observed. Although *Nucleophaga* organisms were found in several species of amoebae (at this location) by Kirby, the one here discussed is of particular interest, not only for its occurrence in a newly described amoeba, but because of a seemingly unique mode of development. In various *Nucleophaga* species, the

nucleolus (chromatin material of the host) is either devoured from within or pushed to the perimeter and flattened against the host nuclear membrane in the process of its utilization. However, in the *Nucleophaga* in *E. disparata*, the coccoid bodies of the parasite develop first around the periphery of the host nucleus (inside, of course, the nuclear membrane), enclosing a ‘central zone’ in which the mass of chromatin (nucleolar) material of the host is for a time ‘contained’ (see Kirby’s, 1927, figs. 40, 43; and Kirby’s, 1941, figs. 218B-E). Eventually, though, the *Nucleophaga* will ‘fill-in’ the nucleus, host nucleolar material being destroyed. Kirby (1932) also observed (with sparse comment) a few examples of a *Nucleophaga* seen in *Entamoeba beamonti*--occurring in another genus of termites (*Amitermes*) found at Barro Colorado Island; the distinction of this additional *Nucleophaga* remains to be determined.

3. *Nucleophaga* sp. (see Sassuchin, 1934). In examining an intestinal amoeba (*Entamoeba citelli*) occurring in *Citellus pygmaeus* (the ‘steppe suslik,’ a type of ground-squirrel) from Russia, Sassuchin (1934) found ‘hyperparasites’ in this amoeba--one of which occurred in (sometimes eventually filling) the nucleus, potentially causing significant hypertrophy. Sassuchin determined this nuclear parasite to be *Nucleophaga*, evidenced by his figs. 5 and 6. Sassuchin did not provide a species name. Kirby (1941) considered ‘Sassuchin’s *Nucleophaga*’ to possibly represented an example of the so-called ‘type-two’ development of *Nucleophaga* (i.e., no multinucleate, membrane-bounded, plasmodial or sporangial structure evident). Sparrow (1960) simply noted Sassuchin’s reference as an example of the numerous mentionings of *Nucleophaga* in protozoological literature. Puzzling, is that Dick (2001) listed Sassuchin’s (1934) reference (thus, presumably the *Nucleophaga* that Sassuchin was observing) under both *Nucleophaga hypertrophica* (therefore also *N. intestinalis*, considered a synonym by Dick) and *N. peranemae*--both species seeming unlikely placements (for Sassuchin’s organism) because of the quite different hosts involved (particularly in the case of *N. peranemae*). It is more likely that Sassuchin’s *Nucleophaga* would prove to be a distinct species, if it could be examined again.

4. *Nucleophaga* sp. (see Kirby, 1941; Karling, 1971). As discussed by Karling (1977), Kirby (1941) noted a probable *Nucleophaga* in *Pseudospora volvocis* (*Pseudospora* being a parasitic, or sometimes ‘scavenger,’ amoeba that may also have a flagellated stage) which had invaded the alga, *Volvox*. As further noted (Kirby, 1941; Karling, 1977), this interpretation (of spore-like bodies of a probable *Nucleophaga* parasitizing nuclei of *Pseudospora*) was based on earlier observations (Robertson, 1905) in which these ‘bodies’ or ‘spheres’ were then interpreted as uniflagellate gametes of *Pseudospora* that were somehow forming within the *Pseudospora* nucleus (not a likely scenario). Roskin (1927), however, clarified the situation to a considerable extent (including by his own observations) in pointing out that the ‘gametes’ (sphere-like structures) seen by Robertson (1905) corresponded generally to structures (small bodies seen in nuclei of hosts) assignable to a Chytridiaceous fungus (a good determination at the time)--not unlike structures observed and so interpreted by other authors in that general time-frame (e.g., Epstein, 1922). Figures 15 and 19 in Robertson (1905) indeed appear to represent *Nucleophaga*, pressing the nucleolus against the nuclear membrane. Her figure 20a shows motile cells, each with a subapically to laterally (i.e., generally anteriorly) attached, somewhat curved, trailing flagellum; her figure 20b illustrates two of these motile cells fused (the resultant cell being biflagellate--a zygote?). So, how to interpret all of this? First, the organism, as concluded by Kirby (1941) and Karling (1977), *probably is* a *Nucleophaga* (occurring, unquestionably, in the *Pseudospora* nucleus). It is probably not truly a chytrid, as would now be understood--chytrid zoospores (and gametes) being posteriorly flagellate (i.e., a precise posterior flagellar attachment). There are though some forms, such as *Olpidiomorpha*--that *may* prove to belong to the Cryptomycota--in which a subapical (at least somewhat anterior) attachment of a trailing flagellum occurs; this is probably also true for species of *Sphaerita* (cf. Sparrow, 1960). So, in the case of what Robertson (1905) observed, and Roskin (1927) and Kirby (1941) more correctly interpreted, one could make a case that the motile structures seen by Robertson were possibly gametes [zoospores?], not of *Pseudospora*, but of a ‘cryptomycotaceous fungus’--*Nucleophaga* being a good possibility. So, why is this of significance? It is so because, if correctly interpreted by Kirby (1941) and Karling (1977), this is the only example of motile cells found in *Nucleophaga*, and the only instance of potential sexual

reproduction in the genus. It would be especially interesting to study this organism again, and confirm the existence of motility in a genus typically disseminating only by non-motile spores (endospores).

5. *Nucleophaga* sp. (see Kirby, 1941). *Trichonympha* is in Family Trichonymphidae (see Lee, 1985b), a group of ‘hypermastigote’ flagellates related to Family Devescovichidae, and more broadly to Trichomonads (see Hall, 1953; Dyer, 1990; Blackwell and Powell, 2006; see also our mention of Devescovichids under #6, List One--and, below, in this listing). Kirby (1941, pp. 1059-1063) noted--in *Trichonympha* found in termites, *Procrystotermes*, studied in Madagascar and in Java--the occurrence of a parasite with *Nucleophaga*-like development. In this case, chromatin material of the host-nucleolus became outwardly displaced (by development of the *Nucleophaga*) as a more or less continuous “peripheral reticulum” (Kirby’s fig. 220A,H) before disappearing--reminiscent of development in *N. hypertrophica* (List One, #3); this pattern seems the reverse of the *Nucleophaga* discussed under #2 (this list). Also, in the putative *Nucleophaga* now discussed (#5), what seemed to be an amoeboid body (early in development of the *Nucleophaga*) penetrated the host (*Trichonympha*) chromatin mass (i.e., nucleolus), possibly bearing some similarity to the plasmodial ‘infection projection’ illustrated by Karling (1977, p. 31, fig. 3)--see also mention of such in the third paragraph of our Discussion. Kirby (1941) noted that this apparent *Nucleophaga* (i.e., #5) is potentially distinctive. This organism should, if possible, be recollected and reinvestigated with present techniques. Fungal parasites of ‘hypermastigote flagellates,’ such as *Trichonympha*--and also ‘devescovichid flagellates, such as *Caduceia* (#6, Lists One and Two)--should, as a matter of generally increasing knowledge of parasites of protozoa, be further inventoried.

6. *Nucleophaga* sp. (see Kirby, 1941). Kirby (1940) first mentioned, and later (Kirby, 1941) more fully discussed and illustrated (pp. 1055-1056), a *Nucleophaga* found in genus, *Caduceia*--a member of the Devescovichid Flagellates, a group of flagellates which are mainly commensalistic or mutualistic, ‘xylophagous’ organisms (Kudo, 1966) living in the intestines of kinds of termites, assisting (with the aid of bacteria) in digestion of microscopic bits of wood. Devescovichids are characterized (typically) by three anterior flagella, and one additional, often different appearing, trailing flagellum (cf. Hall, 1953); they thus are more broadly considered ‘Trichomonads.’ The nucleus is sometimes non-spherical (often cone-shaped or elliptical in *Caduceia*), anterior in the cell, and located at a specific position in relation to the flagellar apparatus, axostyle and parabasal body (see illustrations of various ‘devescovichids’ in Lee, 1985a). These things are pointed out here because--when infected by *Nucleophaga*, and due to structural limitations on uniform expansion of the nucleus--a peculiar hypertrophy (involving unilateral bulging, bending or contortion) of the *Caduceia* nucleus may occur (see Kirby’s figs. 218K,L,N, p. 1055). Kirby’s illustrations of spores (figs. 218 L,N) are interesting in that they appear to be on the large side for a *Nucleophaga*, but are perhaps more interesting because of his illustrations (L,M) of a distinct cytoplasm of each spore, the nucleus becoming eccentric in position. This seems to be a unique *Nucleophaga*, although *Nucleophaga* occurs in a range of Devescovichids (Kirby, 1941); parasites of Devescovichids should, if possible, be evaluated in the light of molecular and electron-microscopic techniques.

7. *Nucleophaga* sp. In their publication, “The Biotic Association of Cockroaches,” Roth and Willis (1960) mentioned--noting Mercier’s publication (1907)--the occurrence of a fungus (*Nucleophaga*) in the nucleus of an amoeba, *Endamoeba blattae*, found in the lower digestive tract of certain cockroaches (e.g., *Blatta orientalis*, the oriental cockroach; and *Blattella germanica*, the German cockroach). Mercier (1907) was convinced of the identification of the nuclear parasite in *Amoeba* (= *Endamoeba*) *blattae* as *Nucleophaga* Dangeard, but seemed less certain that this ‘species’ (unnamed) was the same as Dangeard’s *N. amoebae*. Dobell’s (1919) review of the literature of potentially parasitic protozoa had also yielded mention of the occurrence of *Nucleophaga* in *Endamoba blattae* (citing Mercier’s longer, 1910, publication). Kirby (1927) considered *Endamoeba blattae* distinct from *E. disparata* (#2, this list), though perhaps resembling it in early stages; *E. blattae* eventually demonstrates a distinctive, striated endoplasm (Kudo, 1966, fig. 190d)--more unusual than striated ectoplasm (for examples of the latter, see species of *Thecamoeba*; Bovee, 1985, pp. 172-173). Being found in different (distinct) amoeba hosts--and

(in this case) in different insect hosts--the *Nucleophaga* 'species' respectively in *E. blattae* and *E. disparata* are perhaps different, and should be reinvestigated. A *Nucleophaga* was also observed by Kirby (1927; his plate 26, fig. 61) in *Endamoeba simulans*. However, Kirby considered *E. simulans* similar to *E. blattae*--these two amoebas possibly housing similar *Nucleophaga* species.

LIST THREE: Organisms (or structures) considered, at one point or another, to possibly be *Nucleophaga*, but which we believe should be excluded from the genus.

1. Certain authors (e.g., Kirby, 1941; Dick, 2001) have appeared to conclude that Doflein (1907) observed an organism assignable to *Nucleophaga*. Karling (1977, p. 32) stated that Doflein (1907) "described and illustrated" motile cells (spores? gametes?) from "parasitized nuclei" of an amoeba (in this case, "*Amoeba vespertilio*")--these motile cells said to each possess an 'anterior,' short flagellum, and that such motile cells could fuse in pairs to form [both] biflagellate and amoeboid zygotes. The 'anterior' attachment of the flagellum of this 'organism' might seem reminiscent of the organism discussed by Kirby (1941; see #4, List Two). However, the small [short] forwardly curved flagellum described by Doflein (1907) on motile cells he observed does not match well with what is known of flagella of motile cells of either Chytridiomycota or Cryptomycota, which--though in some cases differing from each other in the point of flagellar attachment--are (in both groups) usually relatively long, and more or less trailing. Both Sparrow (1960) and Karling (1977) questioned if there was enough evidence to consider the organism described by Doflein (1907) a *Nucleophaga*. Dick (2001) listed Doflein's (1907) reference under *Nucleophaga peranemae* Hollande & Balsac, seeming to indicate he thought the organism Doflein observed (not named) would somehow prove (if named) to be a synonym of this species (but, *N. peranemae* spores are not known to be motile!). In any case, contrary to Karling's indication, we could not find that Doflein (1907) illustrated the motile cells he described. In examining Doflein's illustrations (his figs. 2 and 3, p. 17) of the microscopically 'granular appearing,' sometimes irregularly lobed, [non-flagellated] 'organism' he observed in *Amoeba vespertilio*--shown as occupying a significant portion of the trophic, amoeba cell--it seems unwise to assert that this is *Nucleophaga*; it does *not*, in fact, appear to be--although its true nature remains uncertain. It is possible that the 'organism' seen by Doflein (1907) was a rather large, supra-nucleolar, somewhat irregular, lobed, chromatin mass ('reticulum') of the amoeba; see discussion of a "chromatin reticulum" in *Amoeba vespertilio* (Calkins, 1933, p. 96). It is feasible, thus, that Doflein (1907) illustrated an internally 'diffuse,' pre-division stage of this amoeba--in which 'projections' of chromatin extend toward the periphery. In any case, the nature of the motile cells discussed by Doflein is unclear; these may have nothing directly to do with the amoeba in question, or anything parasitizing it.

2. Scherffel (1902), in perusing literature containing incidental findings of 'new' microscopic organisms, noted the occurrence (previously discussed and illustrated by Wildeman, 1898) of a possible *Nucleophaga* in the filamentous, conjugate alga, *Zygnema*. Scherffel's (1902) account (of *Zygnema* allegedly containing *Nucleophaga*) was noted by Sparrow (1960). However, comments in both Scherffel (1902, p. 106) and Wildeman (1898, p. 118) suggest that another organism (potential parasite or scavenger?)--in addition to the supposed *Nucleophaga*--may have been present in the *Zygnema* 'host.' A telling point is that the nucleus of *Zygnema* was not seen at the same time as the parasite (or parasite complex)--unless it is the apparently deteriorated structure in Wildeman's fig. 3--indicating that the algal-cell nucleus had, in most cases, already been eliminated, and, accordingly, that this *Zygnema* 'host' was likely in a 'declining' state (substantiated by the often degenerated appearance of the *Zygnema* plastids; see Wildeman's figs. 1-5). Our further examination of Wildeman's plate 22 (figs. 1-7) suggests that [what appears to be] a protozoan (its apparent cysts capable of attaining larger size than a *Nucleophaga*) had invaded the, perhaps already 'damaged,' *Zygnema* cells; furthermore, the protozoan--possibly a *Pseudospora*, a *Hyalodiscus*, or a *Vampyrellidium* (see, for example, Hall, 1953, pp. 222, 225)--was at some point invaded by a small, apparently fungal, parasite. The fact that this fungal parasite (sphaeroidal at first, e.g. Wildeman, figs. 2, 3, 5) can apparently attain an oval or elliptical shape (cf. Wildeman, figs. 1, 4), and may occur at various places in (even throughout) the protozoan cyst (e.g., Wildeman's figs. 1-3),

additionally indicates that the ‘fungal organism’ is not *Nucleophaga*, but more likely *Sphaerita* (see accounts of the morphology and occurrence of these genera in Sparrow, 1960; Karling, 1972, 1977). In any event, *Zygnema* does not seem to have been *directly* parasitized by the fungal organism in question. *Nucleophaga* is still not known, with certainty, to parasitize organisms other than types of protozoa.

3. Any truly cytoplasmic parasite, such as *Sphaerita* (and *Morella*, if recognized--cf. Karling, 1972--a non-flagellated entity otherwise resembling the allegedly flagellated *Sphaerita*), should not be considered equivalent to *Nucleophaga*. Various authors (e.g., Tyzzer, 1920; Karling, 1972, 1977) appeared to discuss such genera as *Nucleophaga* and *Sphaerita* as almost ‘interchangeable’ entities--assumed to differ mainly by ‘position of occurrence’ in the host-cell (nucleus vs. cytoplasm). Even as regards occurrence in ‘kinds of’ potential hosts, this presumption of ‘essential similarity’ (of *Nucleophaga* and *Sphaerita*) is encountered (e.g., Mattes, 1924; Sassuchin, 1934; Orenski, 1966; Sleigh, 1989). Sleigh, for example, did not clearly distinguish in precisely which organism(s) *Nucleophaga* occurred vs. that in which *Sphaerita* was found. However, the more that various organisms have been investigated, the more it has become apparent that the particular parasitic mode of ‘nucleophagy’ (vs. ‘cytophagy’) is a specific adaptation, involving utilization of the nucleolus in the case of *Nucleophaga* (Michel et al., 2009a, 2012; Corsaro et al., 2014, 2016). Some earlier authors (e.g., Lavier, 1935a) seemed to understand the obligate, unique relationship of *Nucleophaga* to the host nucleus. In any case, it is increasingly clear that what is *truly* a *Nucleophaga* (true nuclear parasite) cannot be equated to species of *Sphaerita* (or *Morella*).

DISCUSSION

As for species of *Nucleophaga*, we exclude (List Three) from the genus only those ‘organisms’ of which we feel certain of their exclusion. All named species (List One), in the current absence of convincing evidence to the contrary, are accepted for now as potentially distinct entities, as are additional forms (List Two, each there designated “*Nucleophaga* sp.”). Contributing to our decision to recognize these ‘taxa’ (lists one and two) is the belief by various authors (e.g., Sassuchin, 1934; Kirby, 1941; Karling, 1977)--with which we agree--in a degree of host specificity of *Nucleophaga*; i.e., different hosts (different amoebae, and certain other protozoa) may house different species of this nuclear parasite. There are probably a number of [perhaps many] species of *Nucleophaga*, although they may indeed resemble one another (cf. Hollande and Balsac, 1941). Such a view would seem supported by the determination (Corsaro et al., 2016) that *Nucleophaga terricolae* and *Nucleophaga amoebae* (cf. Corsaro et al., 2014)--though quite similar in appearance (especially under the light microscope)--are nonetheless molecularly distinct (5.5 % genetic difference). Following this logic, we are, for example, considering *Nucleophaga intestinalis* (Brug, 1926) and *N. hypertrophica* (Epstein, 1922) as potentially distinct, even though various authors (e.g., Brumpt and Lavier, 1935; Dick, 2001) considered them the same (and that *N. intestinalis* was synonym of *N. hypertrophica*). Part of why we do not, presently, accept this synonymy is that Epstein (1922) and Brug (1926) did their original studies of *Nucleophaga* in different parasitic human amoeba--*Endolimax nana* (cf. Epstein), and *Iodamoeba buetschlii* (cf. Brug). We feel it is not possible to be certain that these (and other species we are putatively recognizing) are identical; also, there may be a difference in the effect of these two parasites on the nucleolus of the host (see #3, #4, List One).

Two types of development have been *alleged* for *Nucleophaga*--discussed by Lavier (1935b), and summarized by Kirby (1941). In the *first* type, the enlarging thallus (multinucleate ‘plasmodium’ or ‘sporangium’) of *Nucleophaga* is bordered externally by a (sporangial) membrane, and there is a more or less synchronous development of spores within the ‘sporangium.’ This is the type of development observed by Dangeard (1895) in *Nucleophaga amoebae* (found in the free-living ‘*Amoeba verrucosa*,’ = *A. proteus* according to Pénard, 1905; cf. Kirby, 1941). This is also the mode of development illustrated by Kirby (1941, his fig. 218B-E, p. 1055) for *Nucleophaga* sp. found in *Endamoeba disparata* (occurring in kinds of termites; Kirby, 1927), and appears to be that illustrated by Hollande and Balsac (1941, p. 40, fig. 6) for *Nucleophaga peranemae*. In the *second* type of development, there is *seemingly* no (membrane-

bounded) multinucleate structure (i.e., no ‘plasmodium’ or ‘sporangium’ *per se*), and the spores are apparently the product of non-synchronous divisions. This ‘second type’ is said to be that observed by Epstein (1922) and by Brug (1926) in two different human amoebal parasites, and by Sassuchin (1934) in an amoeba (*Entamoeba citelli*) found in ground-squirrels (see Kirby, 1941, p. 1054). Kirby (1941), though, questioned that there could be two such distinct developmental patterns in *Nucleophaga*--raising the possibility that this apparent difference might be a consequence of misinterpretations, or differing interpretations, of what was seen [or, we might add, whether observations were made from stained slides or living material]; also, the exact stage of the *Nucleophaga*, when observed, could play a role in precisely what was concluded. It is plausible there is merely a spectrum of variation, based on one developmental theme. As regards variation, Lavier (1935b) noted that development in *Nucleophaga ranarum* (in amoebae of amphibians) was similar to the first type of development, *except* that formation of spores was non-synchronous (as in the second type of development)--in other words, an example exhibiting aspects of both supposed types of development. In our opinion, an organism [*Nucleophaga*, or any other], regardless of differences in life cycle, would necessarily possess a plasma membrane.

As regards (the activity of) amoeba hosts, freed spores of *Nucleophaga* are apparently engulfed by phagocytosis (forming a ‘food vacuole’ around them)--much as any potential food particle (Sassuchin, 1934; Karling, 1977; Corsaro et al., 2014, 2018). These ‘engulfed spores,’ however, are not digested, subsequently ‘migrating’ (more likely carried passively, as by protoplasmic streaming) to the host nucleus, penetrating the nucleus and perhaps also the nucleolus (Karling, 1977, attributed this observation to Dangeard, 1895). Karling (1977, fig. 3, p. 31) illustrated an apparently labile ‘infection projection’ [as we term it] from the spore’s cytoplasm. There is also some suggestion, however, that an ‘early plasmodial stage’ may penetrate the nucleus--see Kirby’s (1941) discussion of Greeff (1866); see also Lavier (1935b, p. 357) concerning what is possibly an early plasmodial invasion stage. In any event, the precise sequence of events, once *Nucleophaga* is within the nucleus of the host, has been the subject of discussion--probably involving some variation (differences). Brug (1926) stated that, in the case of *N. intestinalis*, *Nucleophaga* developed *beside* the nucleolus, subsequently flattening it (or its remains) against the nuclear membrane; this also seems to be the case in the *Nucleophaga* sp. found in *Pseudospora* (see Karling’s, 1977, fig. 22, p.31), as well as that observed by Tyzzer (1920, his fig. 14) in *Pygolimax*. Concerning an amoeba in termites, Kirby (1927) observed that the *Nucleophaga* parasite, when within the nucleus of the amoeba, first surrounds the nucleolus, ‘centralizing’ it, before its eventual destruction; however, the reverse of this process, i.e., a ‘peripheralization’ of chromatin (nucleolar material), seems to occur in the case of the *Nucleophaga* found in *Trichonympha* (Kirby, 1941), and perhaps also in *Nucleophaga hypertrophica* (Epstein, 1922, see his fig. 6). Regardless of the precise mechanism utilized by various *Nucleophaga* species, it seems that chromatin material [of the nucleolus of the host] is eventually depleted or eliminated (Kirby, 1941). Some insight into the details of nucleolar depletion is found in Corsaro et al. (2014, 2016) in which it was confirmed the *Nucleophaga* parasite grows at the expense of the host karyosome (= endosome = nucleolus); in fact, minute finger-like extensions were observed on a ‘trophic stage’ of *Nucleophaga* (preceding the ‘endospore stage), indicative of the ability of *Nucleophaga* (albeit perhaps in a ‘residual’ form) to perform phagocytosis; remnants of host nucleolar material were found inside the *Nucleophaga* cytoplasm (Corsaro et al., 2014; see their figs. 1d,h).

Nucleophaga was, from the time of its description (Dangeard, 1895), considered a chytrid-like organism. In spite of being considered ‘fungal,’ *Nucleophaga* was omitted by Fitzpatrick (1930), Clements and Shear (1931) and Bessey (1950)--odd omissions, since these authors included *Sphaerita* (a genus thought similar to *Nucleophaga*, but which infects cytoplasm of the host rather than the nucleus; cf. Sparrow, 1960). When *Nucleophaga* was included in coverage, it was typically placed in family Olpidiaceae (Chytridiales)--see Sparrow, 1960 and Karling, 1977--near *Sphaerita*. Both Sparrow and Karling (see also Karling, 1972), at some point, questioned the generic relationships of *Nucleophaga*. Dick (2001) merely included *Nucleophaga* among a mélange of “miscellaneous genera”--some very possibly unrelated. Corsaro et al. (2014), based on molecular and microscopic evidence, considered

Nucleophaga to represent a distinct lineage within the Cryptomycota (viz. “Rozellomycota” of Corsaro et al., 2014)--having morphologically, though in diminished form, a *Rozella*-like trophic stage (see p. 4494 in Corsaro et al., 2014). This viewpoint was modified in Corsaro et al. (2016) in which the “Rozellomycota” and the Microsporidia were suggested to be evolutionarily connected; *Nucleophaga* seemed to fit into this revised picture as a somewhat intermediate [morphologically and genetically] entity, which, based on updated molecular findings, formed a sister clade (rather obscurely named “Nucleophagales,” cf. Corsaro et al., 2018, their “Introduction”) with Microsporidia--being perhaps more distantly related to *Rozella*. Possible further clarification came when Bass et al. (2018) determined *Nucleophaga* to be a distinct lineage within an expanded (diverse) Microsporidia; not just molecular evidence, but TEM studies (demonstrating such microsporidian features as polar filaments, an anchoring disc, loss of flagellation, and lack of mitochondria) appeared to confirm this (Corsaro et al., 2016; Bass et al. 2018); *Rozella* (by contrast) has mitochondria--although depauperate in the thallus (James et al., 2013), compared with the more robust, single mitochondrion in the *Rozella* zoospore (Held, 1975). Bass et al. (2018) viewed the Rozellida as a more restricted group (centering mainly on *Rozella*) than, for example, did Corsaro et al., 2014, 2016). Some authors (e.g., Corsaro et al., 2018), however, continued to view the “Rozellomycota” more broadly--considering it to encompass Microsporidia, *Nucleophaga*, and *Rozella* (*Rozella* connected basally to both the preceding)--suggesting as well that Rozellids constitute an early chytrid lineage, and that Microsporidia also evolved from Rozellids (see James et al., 2013, re: the potential connection of Microsporidia and Cryptomycota). In any case, *Nucleophaga* has closer relationships to Microsporidia and Cryptomycota than to Chytridiomycota (i.e., as presently recognized). It has recently been suggested (Blackwell et al., 2016, 2017) that certain other genera, e.g., *Plasmophagus* and *Dictyomorpha*--once considered to be in family Olpidiaceae (Chytridiales), along with *Nucleophaga* and *Sphaerita*--may as well have closer relationships to Cryptomycota than to Chytridiomycota.

Corsaro et al. (2018) pointed to the need for continuing study of amoebophagous organisms (such as *Nucleophaga*), as these are likely to prove key to understanding early evolution of fungal groups, and primitive animals (i.e., kinds of ‘protozoa’). Not only molecular studies (e.g., Corsaro et al., 2014, 2016; Quandt et al., 2017; Bass et al., 2018), but those of electron microscopy (e.g., Michel et al., 2009a, 2009b, 2012) are important to ascertain the precise nature (even proper identification) of the organisms, the mode of action (exact nature of the parasitism), and the life cycles of such primitive forms (how they are able to survive and function in their hosts)--explaining such facts as, for example, that *Nucleophaga* has morphological/functional characteristics of both *Rozella* (e.g., phagocytic ability, involving small pseudopodia; Powell et al., 2017) and of Microsporidia (e.g., polar filaments and adhesions discs; discussed above). It is important to understand that *Nucleophaga*--though quite small (0.4 to 2.5 μm), and typically with no more than slight mobility of its own, at any point--does indeed have a ‘trophic stage’ (elucidated by electron microscopy; see Corsaro et al., 2014, 2016) preceding the more familiar ‘endospore stage;’ this had proved essentially impossible to determine with light microscopy, because of the tiny and seemingly uniform nature of the coccoid bodies of the parasite. *Nucleophaga* may now be understood as a kind of morphological ‘missing link,’ as well as representing ‘molecular intermediacy,’ among groups within which its relationships are now understood to lie (see preceding paragraph). Needed are more studies (such as those of Corsaro et al. and Michel et al., mentioned above) of additional taxa of *Nucleophaga*--and, of possibly related organisms such as *Sphaerita* (and even *Morella*, which has never been nomenclaturally validated, cf. Karling, 1972, 1977; and which may also be a later homonym, cf. Dick, 2001). Such desired studies invoke the necessity of, first, collecting and screening of protozoan hosts potentially containing new or poorly known parasitic (or ‘hyperparasitic’) organisms.

It would be well to re-collect any entities discussed in our Lists (One, Two) that potentially represent taxa additional to the two species (List One) already well-studied by Corsaro et al. (2014, 2016; molecular analysis). Further possibilities for study, perhaps less well known, are sporadically found in the literature--e.g., Ghosh (1970) who noted the find (Wenrich, 1940) of a possible *Nucleophaga* in *Entamoeba muris* (occurring in the caecum of mice and rats), and Garcia (2007) who appeared to indicate

occurrence of *Nucleophaga* sp. in *Entamoeba coli* (in humans). It can admittedly sometimes be difficult, especially in older literature, to distinguish the presence of *Nucleophaga* from what may be merely a division preparatory stage of the host organism, e.g., in which so-called ‘chromidia’ are found, or even odd, stained, ingested particles (see examples, figs. 89E and 90B of *Entamoeba coli* and *E. histolytica* respectively, in Minchin, 1922). Regardless, any newly found (or ‘found again’) taxa of *Nucleophaga* should be reported, even if left unnamed. Also, better understanding of the life cycle of *Nucleophaga* may accrue from such investigations. That nucleophagy (especially involving utilization of the nucleolus) is a specific adaptation--pointing to distinction of *Nucleophaga* as a genus--has been discussed (List Three, #3). However, as mentioned, the details of nucleolar utilization may vary between (perhaps even within) species. The rare occurrence of *Nucleophaga* in the cytoplasm (cf. Kirby, 1941, pp. 1053-1054; Karling, 1972, p. 227)--in a more ‘separated’ arrangement than the typically compact (‘morula’) configuration--probably means, due to parasitism (and hypertrophy) of the nucleus by *Nucleophaga*, that the nuclear membrane has broken down (become “invisible,” Sassuchin, 1934, p. 218); the *Nucleophaga* spores, ‘released’ from the nucleus, are thus consequently in (perhaps appearing to have originated in) the cytoplasm (cf. Tyzzer, 1920, p. 204; Brug, 1926, p. 467; Kirby, 1927, p. 197). In other words, it is during this stage that *Nucleophaga* spores are in the initial ‘process’ of being ‘dispersed,’ albeit passively--subsequently entirely from a [dead or dying] host undergoing [eventual] disintegration (Sparrow, 1960, p. 123). Further observations of life cycles of *Nucleophaga* and *Sphaerita* (and related organisms) are needed to determine, with certainty, aspects of development and successive events (e.g., dispersal).

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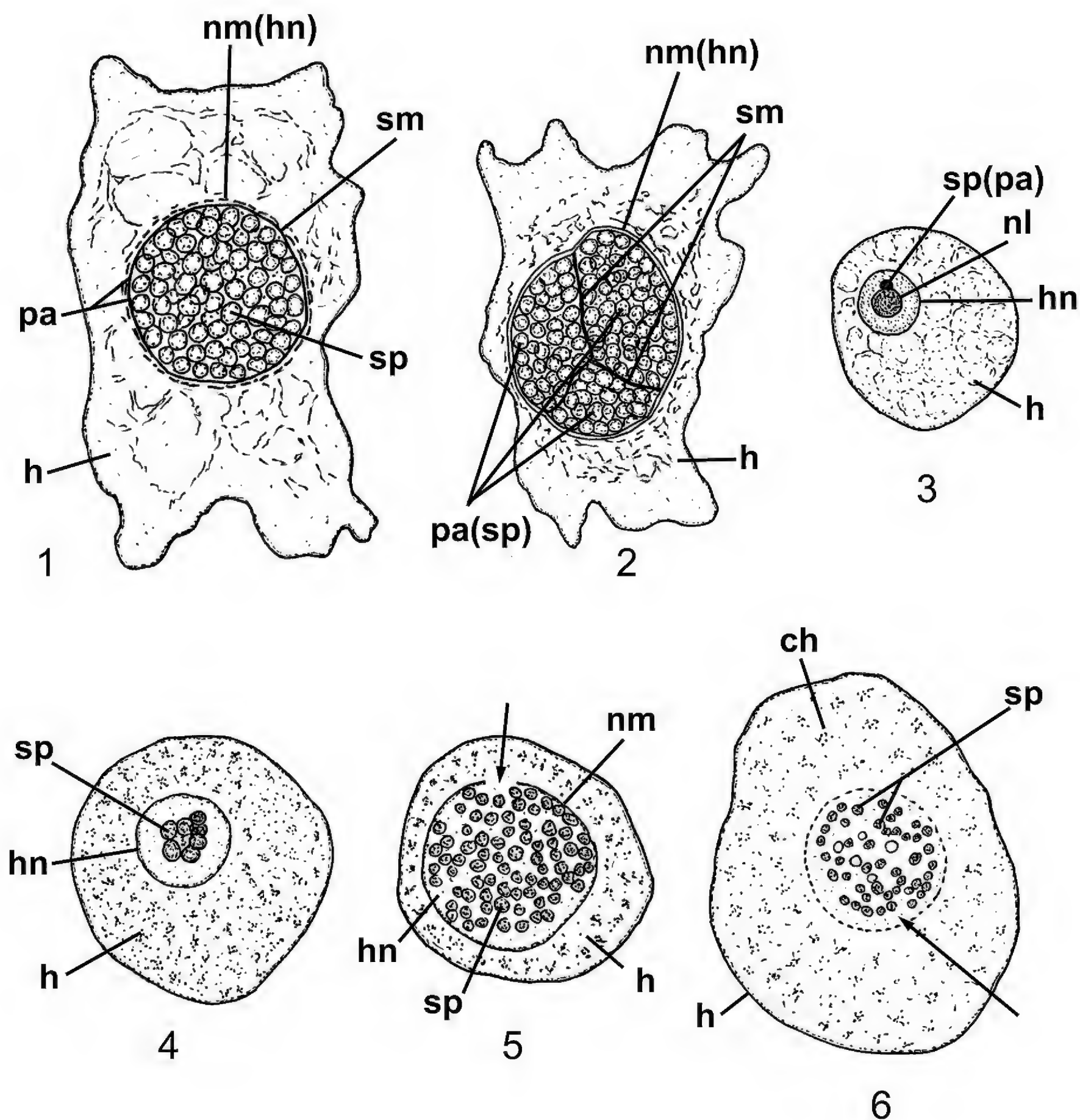
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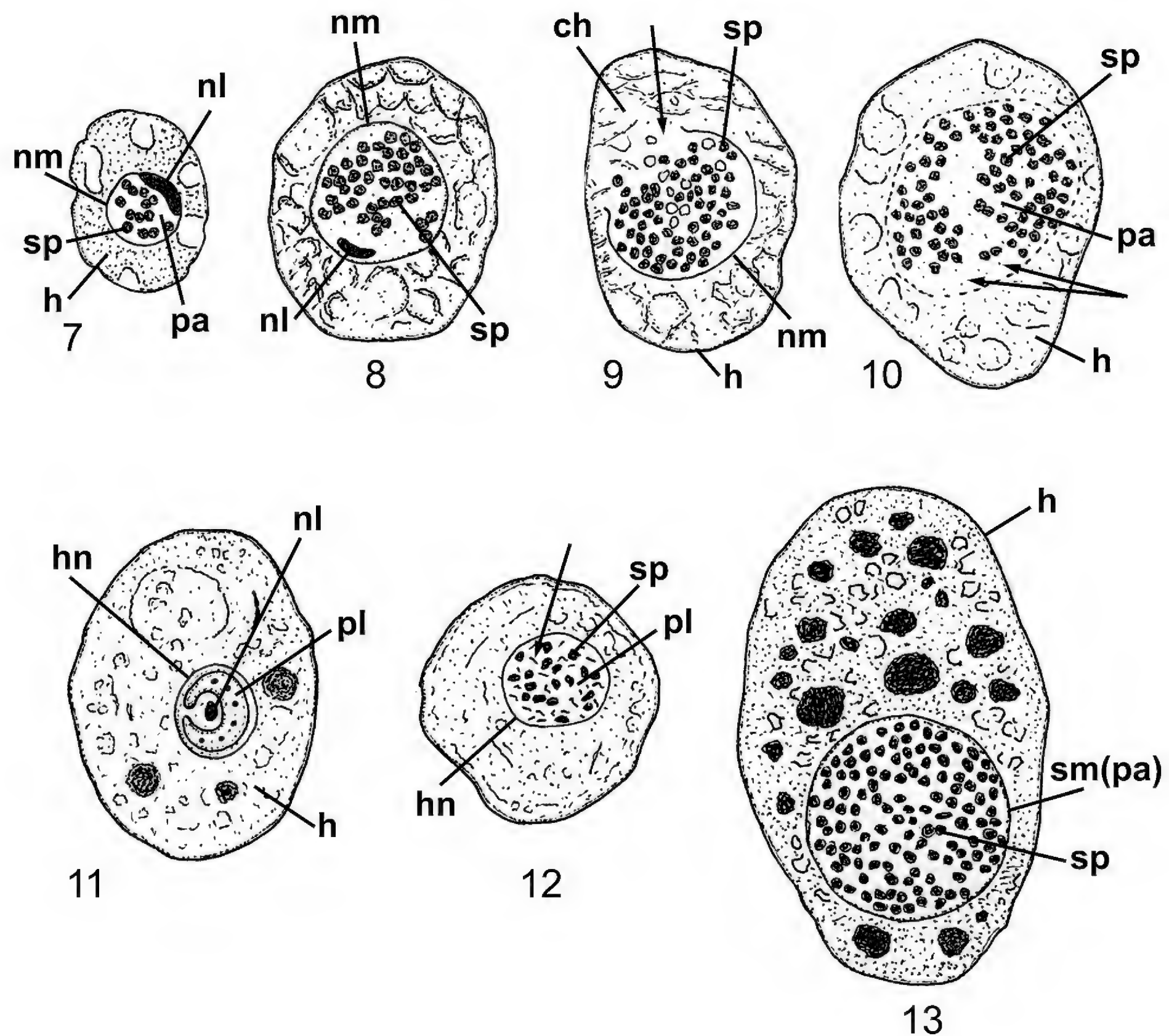
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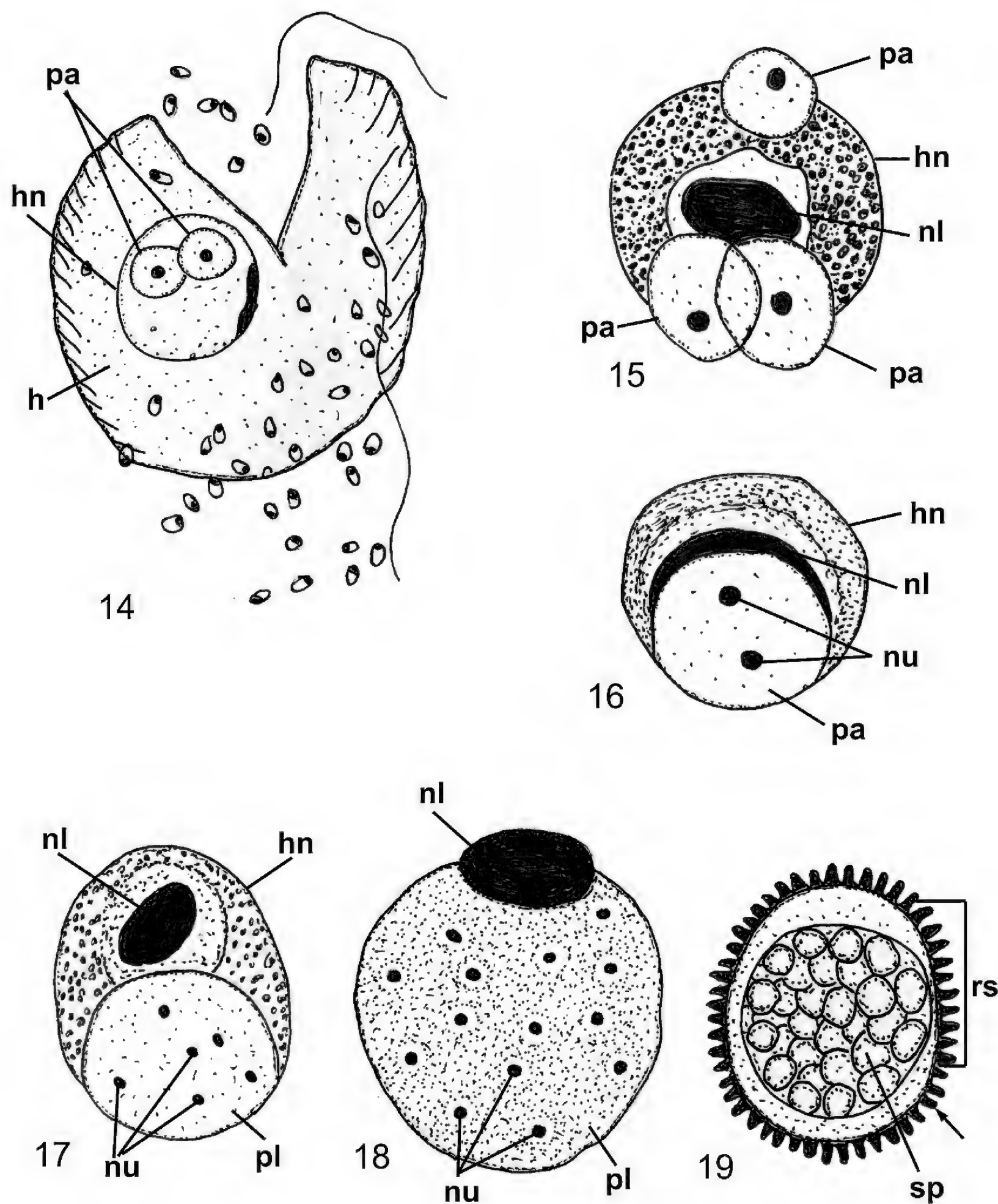
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Figures 1-2. Parasite (pa), *Nucleophaga amoebae* (after Dangeard, 1895); host (h), *Thecamoeba verrucosa*. **Fig. 1:** Coccoid bodies are spores (sp), i.e., endospores of the parasite; note sporangial membrane (sm) of the parasite. Host nucleus (hn) represented by remnants of its nuclear membrane (nm). **Fig. 2:** Three parasites, tightly packed--each with its mass of spores (sp) and sporangial membrane (sm)--occupying hypertrophied host-nucleus (hn) of which only its membrane (nm) remains. **Figures 3-6.** *Nucleophaga hypertrophica* (pa), after Epstein (1922); host (h), *Endolimax nana*. **Fig. 3:** Spore (sp) infecting the nucleolus (nl), inside nucleus (hn) of host. **Fig. 4:** Small group of spores (sp) has arisen, centrally, obliterating the nucleolus. Nucleus (hn) of host beginning to undergo hypertrophy. **Fig. 5:** Mass of spores (sp) now occupies the significantly hypertrophied host nucleus (hn); note ruptured (arrow) nuclear membrane (nm) of host; relative area of host reduced. **Fig. 6:** Group of (endo-) spores (sp); nucleus (including its membrane) of host obliterated; some 'clear space' (arrow) evident around mass of spores; no sporangial membrane present; host cytoplasm (ch) seemingly intact, but shows hypertrophy.



Figures 7-10. Parasite (pa), *Nucleophaga intestinalis* (after Brug, 1926); host (h), *Iododamoeba buetschlii*. **Fig. 7:** Relatively young stage of parasite (but no sporangial membrane evident); spores (sp), infecting host nucleus (nuclear membrane, nm, present--nucleolus, nl, flattened against it). **Fig. 8:** Somewhat older; number of spores (sp) increased. Host nucleus hypertrophied, represented by the nuclear membrane (nm) and flattened nucleolus (nl). **Fig. 9:** Nucleolus no longer evident. Note rupture (arrow) of nuclear membrane (nm) of host and passive release of endospores (sp) into cytoplasm (ch) of host. **Fig. 10:** Older; host nucleus destroyed. Note clear space (arrows) potentially 'separating' parasite and host (sporangial membrane lacking around spores, sp). **Figures 11-13.** *Nucleophaga ranarum* (pa), after Lavier, 1935b; host (h), *Entamoeba ranarum*. **Fig. 11:** Young plasmodial-like structure (pl)--an apparent early infection stage--surrounding remains of nucleolus (nl), inside nucleus (hn) of host. **Fig. 12:** nuclei of 'plasmodium' (pl) converted to spores (sp); pieces (arrow) of interior of host nucleus (hn) scattered. **Fig. 13:** Older; nuclear area hypertrophied; nucleus destroyed. Sporangial membrane (sm) of parasite still evident. Spores (sp) 'multiplied' in presumably non-synchronous fashion. Host generally hypertrophied.



Figures 14-19. Parasite (pa), *Nucleophaga peranemae* (after Hollande and Balsac, 1941); host (h), *Peranema trichophorum*. **Fig. 14:** Two smallish parasites developing in host-nucleus (hn). **Fig. 15:** Illustrates only the nucleus of the host (hn), nucleolus (nl) inside, three young parasites developing. **Fig. 16:** Host nucleus (hn), with single parasite inside--i.e., an incipient plasmodium which divided (internally) forming two nuclei (nu). 'Dark cap' is host nucleolus (nl) flattened, pushed toward one side. **Fig. 17:** Developing plasmodium (pl), several nuclei (nu) now evident. As may be judged, the exact stage at which the nucleolus (nl), of the host-nucleus (hn), is flattened or pushed aside, varies. **Fig. 18:** Only the parasitic plasmodium (pl) shown; more nuclei (nu) now evident, each potentially becoming a spore; nucleolus (nl) of host-nucleus in process of being flattened, but persisting for a time. **Fig. 19:** Resting spore (rs) or 'cyst,' note spore-wall with short spines (arrow), and note mass of spores (sp) internally.